STANDARD OPERATIONG PROCEDURES

FOR SEMEN PROCESSING

LABORATORIES IN INDIA

CENTRAL MONITORING UNIT

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Chapter – 1

Standard Operating Procedures for Bull Management

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The Standard Operating Procedures (SOP) provides general guidelines for all the activities that are taking place in a Frozen Semen Station (FSS). All the guidelines may not be appropriate in all the situations / conditions. The respective FSS has to prepare a few more guidelines supplementing the SOP.

Bull Management:

Introduction

Breeding bulls selected by Frozen Semen Station (FSS) are considered highly valuable units of semen production. Good management practices are essential for maximizing use of the selected bull. The breeding bull is expected to produce large number of frozen semen doses (FSD) of good quality which will result in optimum pregnancy rate in the field. The number of doses produced per bull per year depends on breed, size and age of the bull.

Bull management comprises of following:

1. Supply of adequate fodder and feed.
2. Supply of potable water at all the time.
3. Housing and floor
4. Exercise of bulls
5. Vaccination, screening and measures for health care

1. Supply of adequate fodder and feed.

The management of fodder and feed for breeding bulls is not as complicated as that of dairy cattle. The requirements for maintenance if met are adequate. However the regularity and consistency in the quality is an important factor.

a. Supply of fodder

The bulls being ruminants require fodder of good quality and in adequate quantity for their maintenance. It is estimated that a bull requires 2.5 – 3.0 % of body weight as dry matter per day. This includes concentrate i.e. compounded cattle feed. A table showing requirements of nutrients may be referred for providing fodder and feed.

The quality of fodder of any crop varies considerably according to age of the crop, soil conditions and irrigation etc. Hence it is recommended that each FSS should arrange proximate analysis of different fodder crops cultivated in order to determine the nutrient quality and optimum time of harvest of fodder.

Feeding Growing and Mature Bulls

Daily nutrient requirements of growing and mature bulls *							
Body wt (Kg.)	**gain/day (g)**	**DM/day (Kg.)**	**C.P. (g)**	**TDN (kg)**	**Ca (g)**	**P (g)**	**Vit. A (1000 IU)**
Growing bulls							
100	750	2.8	390	1.9	11	8	4
150	750	4.3	460	2.7	15	11	6
200	750	5.7	530	3.4	18	14	8
250	750	7.0	610	4.0	21	16	10
300	750	8.2	680	4.6	23	17	13
350	750	9.3	760	5.2	24	18	15
400	700	10.2	820	5.7	25	19	17
450	600	10.4	875	5.8	26	20	19
500	400	10.0	885	5.6	26	20	21
550	250	10.0	845	5.6	25	19	23
600	100	9.8	800	5.5	24	18	26
Maintenance of mature breeding bulls							
500	-	8.3	640	4.6	20	15	21
600	-	9.6	735	5.4	22	17	26
700	-	10.9	830	6.1	25	19	30

Daily ration for Bulls						
Body wt. (Kg.)		**Calf starter (Kg.)**	**C.F. (Kg.)**	**B.P.F. (Kg.)**	**Hay (Kg.)**	**Green Fodder (Kg.)**
Growing bulls						
100		2	-	-	0.5	6-8
150		-	-	2	0	8-10
200		-	-	2	0.5	15
300		-		2	1	ad lib.
400	a)	-		2	3	ad lib.
	b)	-	2.5	-	3	ad lib.
500	a)	-	-	2.5	2-4	ad lib.
	b)	-	3	-	2-4	ad lib.
600	a)	-	-	2.5	2-4	ad lib.
	b)	-	3	-	2-4	ad lib.
Mature breeding bulls						
500	a)	-	2.5	-	2-4	ad lib.
	b)	-	-	2	2-4	ad lib.
600		----------do----------				
700		----------do----------				

Except for very tender and succulent fodders like Lucerne / Berseem, all other fodders should be chaffed before feeding. The blades of the chaff cutter should be adjusted in such a manner to ensure the size of the cut fodder is at least 50 mm. Too small size of fodder is not recommended. The dry fodder i.e. hays / Stover should be chaffed preferably along with green fodder. The dry fodder can also be fed separately.

Feeding of good quality hay is recommended instead of feeding paddy straw / maize or Ragi straw. The hay is more nutritious and palatable. Wherever it is possible to feed large quantity of hay, the green fodder can be reduced to 5 – 10 kg / bull / day. The sorghum hay, oat hay and Lucerne hay are most recommended for bulls. As a result of feeding good quality hay in larger quantities, the bulls will be more clean and hygienic.

Feeding of silage under tropical conditions has shown varying results. Feeding of maize / oat silage in limited quantity of 10 – 15 Kg. / day / bull is harmless. Wherever silage is prepared, adequate precautions should be taken to avoid growth of moulds and aerobic condition.

b. Supply of feed

In order to balance the nutrient requirements, it is essential to include limited quantity of cattle feed. The feed should be produced using good quality ingredients free from toxic elements and moulds and fungi. The FSS should arrange for proximate analysis of the feed at regular intervals to ensure the nutrient quality of the feed. The feed should be purchased from a reputed manufacturer or should be prepared in house. The feed can be in the form of mash or pellets. It is preferable to avoid feeding of cotton seed cake to bulls.

The feed should be supplied to bulls mixed with small quantity of green / dry fodder to stimulate salivary glands.

c. Supply of Mineral mixture

Supplementary feeding of 30 – 80 g. of mineral mixture / day / bull is essential to meet the physiological requirements. The quantity varies upon the body weight of the bull and mineral profile of the soil used for fodder cultivation. If data on mineral profile of the district is available, it should be used for formulating appropriate mineral mixture. The ingredients of the mineral mixture should be free from animal origin. The mineral mixture without salt should be preferably purchased. Salt in the range of 10 – 15 g / day / bull can be added to the mineral mixture. The mineral mixture should be thoroughly mixed with concentrate feed to avoid wastage.

2. Supply of potable water at all the time.

a. Water is one of the most important component of nutrients and it should be made available all the time. The water should be clean, free from organic matter and potable. The water should be under shade and accessible all the time.

b. Fresh water should be available all the time.

c. River water / pond water should be used after adequate treatment to eliminate contaminants and biological material.

3. Housing and floor.

Each FSS should have permanent housing arrangements for mature breeding bulls along with facility for isolation shed for about 4 – 5 bulls. The isolation shed should be about 500 mts. from the main housing of bulls. In addition each FSS should also have facility for quarantine of new bulls, which are to be inducted into FSS. This facility should be at least one kilometer away from main housing. All housing facilities should have arrangements for feeding and supply of water.

a. Housing & temperature control

The bulls have to live 24 Hours and 365 days in the housing provided. The type of housing decides the comfort level and quality and quantity of semen production.

The sheds should be in East – West direction to avoid direct sunlight on bulls. There are reports of testicular degeneration due to intense sunlight on testis / scrotum. In case the sheds are already in north – South direction, measures should be taken to provide shade to bulls to protect from direct sunlight. The sheds should be airy and allow breeze. Trees to protect from hot air during summer should surround the sheds. There should be top ventilation to allow escape of hot air. In the states where the climate is hot most of the part of the year, the sheds should have minimum walls and steel pipe railing should be used for better ventilation. There should be provision of water foggers and fans for use during the summer particularly for exotic breeds and buffalo bulls. The fans and water foggers should be operated alternately for about 4 – 5 hours / day during summer time. During summer, it is desirable to wash the buffalo bulls twice a day. The exotic and crossbred bulls can be washed with water on alternate days to keep them clean. It is recommended to groom the cattle bulls with coir / nylon brush every day to keep the skin in shining and glowing condition. The asbestos roofing of the sheds should be painted white on outer surface and black on the inner surface.

Single bullpens having loafing area are suitable and ideal for breeding bulls. The bulls should be kept free. The bullpens should have feeding manger and water trough. Each bull should have adequate covered area (minimum 10 M^2).

In places where the climate is very cool most of the year, the walls are recommended. However attention should be given to ensure adequate ventilation and avoiding stale air in the sheds.

In places where the individual bull housing is not practiced, adequate bedding should be provided.

b. Flooring

Each bull should have adequate loafing area (minimum 10 M^2). The flooring of covered area should be non-slippery cement concrete with adequate grooves. The flooring of the loafing area should be preferably with natural soil. In case of heavy rainfall areas, the flooring can have sub soil drainage system to avoid slush. To avoid excessive slush in monsoon, there should be drain to take away rainwater from roof.

The housing should be designed in such a manner so that the personnel working are safe. If possible the loafing area and covered area should be separated with a gate so that persons can clean. The floors should be scrubbed and burnt at least once in a year by blowlamp.

4. Exercise of bulls.

As per the published literature, the effect of exercise of bulls on the semen quality is varying. However it is preferable to exercise the bulls at least on alternate days for about 45 minutes to one hour. During the exercise, it would be possible to observe the gait and note any lameness / foot problems.

5. Vaccination, Screening and measures for health care.

Considering the physiology of spermatogenesis, it is preferable to avoid any type of vaccination as vaccination induces febrile reaction and thereby quality of semen. However keeping in view the prevailing animal disease outbreaks, it is recommended to plan a vaccination schedule and implement strictly. Wherever possible it is recommended to minimize the number of vaccinations each year using combination vaccines like HS & BQ. In case of FMD, it is preferable to use oil adjuvant vaccine.

It is recommended to wash the site of vaccination with 2% Savlon / Dettol solution prior to vaccination to avoid formation of abscess due to secondary infections. The spot of injections should also be cleaned with surgical spirit / tincture of iodine. One should ensure use of correct vaccine and correct dose and route. The instructions of

manufacturer should be followed and the information batch number, date of expiry of vaccine should be recorded at the time of vaccination.

It is preferable to arrange for vaccination of cloven-footed animals in 3 – 5 Km. radius of FSS. This will help in control of outbreaks in FSS and minimize losses. By this method, it will be possible to establish a ring of immunity.

The breeding bulls should be screened for Brucellosis, TB, JD, Campylobacter and Trichomoniasis. The Government of India has mandated the Regional Disease Diagnostic Laboratories (RDDL) for screening the breeding bulls in the Frozen Semen Stations. The officer In charge of FSS should establish contact with the respective RDDL for deciding schedule of screening. Proper documentation of the screening should be maintained.

Each FSS should have a tyre wash which should be filled with 4% solution of washing soda (Sodium Carbonate). The size of tyre wash should be at least 8.5 Mts. long 3.5 mts. wide and 20 cms deep to ensure dipping of rear tyre of tractor. This is required to avoid entry of viral diseases into cattle sheds. At the place of entry to FSS, a footbath and hand wash should also be provided. The footbath as well as hand wash should have 4% soda solution. Provision for washing of hands after dipping in the soda solution should be made. All the staff as well as visitors to FSS should use foot bath as well as hand wash before entering. The bull sheds as well as pathways should be sprayed with 4% soda solution at weekly intervals to reduce chances of viral diseases.

If ecto parasites are common, measures should be taken to reduce by use of safe sprays like Butox etc. If tick infestation is high, the exotic and crossbred bulls should be vaccinated against Theilerosis once in three years.

In case of buffalo bulls, shaving of the over grown hair at periodical intervals has to be arranged.

The hooves of the bulls have to be trimmed at periodical intervals to prevent over growth and lameness. Particularly bulls of exotic breeds like H.F. are more prone for hoof problems. Wherever pure H.F bulls are maintained, it is recommended to arrange a separate footbath for bulls. The bulls should be made to stand in the footbath for at least half an hour at weekly intervals. The footbath should be filled with 0.5% copper sulphate solution. Alternately 1% formalin solution can also be used either for footbath or spray on the hooves.

Strong smelling disinfectants like phenyl or formalin should not be used in bull shed instead compound like gluteraldehyde, colloidal iodine or chlorine compounds like hypo chlorate be used.

Preparation of bulls for collection:

Each FSS should have the semen collection roster and it should be accessible to the staff working in the bull sheds. The preparation of the bulls scheduled for collection should begin on previous day collection.

The prepucial hair should be trimmed to 2-cm. lengths. Long prepucial hair causes adhering of the dung leading to spoiling the ejaculate. If trimmed too closely, it will cause irritation leading to frequent masturbation / infection of prepuce.

The under side of abdomen should be washed on previous day to ensure freedom from dung etc. An hour prior to collection, the under side of cattle bulls should be brushed with a groom to remove adhering dust / grass. The prepucial opening should be sprayed with 0.9% normal saline solution and wiped dry using a disposable paper napkin. In case of buffalo bulls, as thick hair is not present wiping of under side of abdomen with a dry cloth and followed by cleaning of prepucial opening as mentioned above.

All the bull attendants should wear uniform of same color. The bull attendants who work closely with bulls i.e. grooming, semen collection etc. should wear protective foot wear to avoid injuries.

Chapter – 2

Hygiene and Precautions required by a Semen collector

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Hygiene of collector

a. Before entering the collection arena changes civil dress and footwear in the anteroom.
b. Wear protective semen collection clothing (pilot suit and cap) and gumboots. Greenish coloured overalls are preferred.
c. Before every collection, wash hands with 0.1 % savlon or use disposable gloves or do both.
d. Use a new pair of disposable gloves for each collection. If not, wash and disinfect hands after each semen collection.
e. Use a sterilized napkin to wipe hands and change napkin after each collection.
f. Collector should not enter the AV room during collection.
g. Wash the footwear and coat daily immediately after completion of semen collection work.
h. The semen collector should not enter the semen laboratory

Precautions to be taken by Semen Collector:

1. Collection arena

a. Collection area should have at least 3 to 4 semen collection crates so that the mounting bull will get sufficient choice of stimulus animal. The arena should provide good footing for the bull and the teaser.
b. Ensure that there are no slippery surfaces in the collection area.

2. Collect semen from only a clean and groomed bull

a. Check the coat and underline of the bull to be collected for any dung or dirt
b. If soiled, clean carefully with soap or mild detergent long before collection.
c. Rinse then with clean water and dry with clean towel.
d. Towel used on one animal should not be reused on another.
e. Ensure that animal is dry at collection.
f. The preputial hair of the bull to be collected should be short (2 cm) and hooves trimmed.

3. Provide adequate sexual preparation for the bull

a. Adequate sexual preparation eliminates the need for intensive collection.
b. Select appropriately sized stimulus animal and present it in an area affording the bull good footing.
c. The bull's nose is tender. Therefore the lead rope should not be jerked violently.
d. Novel stimulus elicits sexual response in bulls and hence the collector should attempt novelty through the following approaches:
    1) Presentation of the same stimulus animal in a new location,
    2) Presentation of a new stimulus animal or
    3) Combination of animals in the original location or presentation of new stimulus animals in a new location.
e. Bull should be allowed at least two false mounts with two minutes restraint before taking collection.
    1) During the false mount the bull should be encouraged to mount directly from the rear.
    2) The semen collector should ensure that whenever a bull mounts every effort must be made to assure that the mount animal does not move.
f. Use of bull aprons during false mount will avoid the penis touching hindquarter of the stimulus animal.
g. Tie the bull aprons just before starting the false mount .Use separate bull aprons for each ejaculation and each bull.

4. Collection of semen

a. Semen should be obtained from an animal with normal libido.
b. Collection should be attempted only if the bull is properly sexually prepared.
c. Check temperature of the artificial vagina before collection. Ideal temperature is 45ºC for adult bulls and between 42-45ºC for young bulls. Pressure- 45 to 55 mm of Hg. Younger bulls requires higher pressure.
d. After the penis has completely separated from the sheath serve the AV. The collector should not touch the penis of the bull, the touching of the penis by the collector causes shying of the bull and the bull will not readily mount
e. Ensure that there is an interval of minimum 15 minutes between two collections of the same animal.
f. Be certain that the proper sized artificial vagina is used. Use 30 cm AV for adult bull and 25cm Av for adult buffalo bull
g. Hold the AV before and after each semen collection in such a way that the lubricating jelly/ debris after collection is not mixed with the ejaculate.
h. The collection tube should be kept warm at 35oC before collection. It should be covered with felt cap, or warm water jacket
i. The whole cone and collection tube should be covered with insulation bag. The window of the insulation bag should be always kept on dorsal side.
j. Protect the semen collection tube from sunlight.

k. Never use chipped or defective collection tube.
l. Separate AV should be used for each bull and for each ejaculate and even if the bull inserts the penis in the AV without ejaculation the AV should be changed
m. To reduce possible loss of sperm do not remove the artificial vagina from the penis too quickly after the bull has ejaculated.
n. Avoid contamination of semen by water, lubricating jelly or other harmful substances.
o. Be certain to identify semen collection tube with the correct bull number.
p. Report any abnormal events to laboratory personnel (for example: bloody semen, leak in inner liner, loss of ejaculate).
q. Immediately after collection dip the AV's in a container having neutral detergent solution for cleaning.
r. Teamwork between bull handler and semen collector is essential to assure correct bull identification, use of correct sexual preparation procedure and safety of employees.
s. At the time of collection both the collector and herdsman should avoid sudden moves.
t. Avoid distractions during semen collection. The entry of visitors shall be prohibited.

5. Management of Bulls during collection

a. The semen collector should understand the individual bull's sexual behavior and physiology.
b. The semen collector and bull attendants should not be changed frequently.
c. Never mistreat or abuse a bull at the time of semen collection. Too high temperature of AV, forcing of the AV on penis, grasping of the penis instead of sheath are painful conditions leading to sexual suppression.

Chapter - 3

Standard Operating Procedures (SOP) for Semen processing, Evaluation and Freezing.

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Buffer preparation

a. Is to be done in a separate buffer / dilutor preparation room which has been sterilized regularly by formalin humidification as per MSP
b. All chemicals used for buffer preparation namely:
    1. Tris (hydroxy methyl amino methane)
    2. Citric acid monohydrate
    3. D-Fructose

    Should be procured from reputed manufacturers and should be either of Analytical grade (AR) or Graded reagent (GR).
c. Laboratory Grade (LR) of chemicals should not be used due to low purity.
d. Chemicals should be weighed by an electronic balance for high accuracy in order to arrive at the desired pH and osmolarity.
e. Preferably Milli Q ultrafilteration water (of 18 mega ohms) should be used for preparation of the buffer.
f. Alternatively freshly prepared distilled and autoclaved water shall be used.
g. Stored distilled water should never be used for buffer preparation as heavy metals leach after storage.
h. The buffer should be prepared by a competent person of the laboratory (lab technician / lab assistant)
i. All glass wares and consumables required should be brought in before preparation starts to prevent repeated entry into the room, alternatively a pass box may be installed in between the sterilized glassware room and dilutor preparation rooms.
j. All glass ware should be of borosil / corning quality and sterilized by an hot air oven at 161 degree Celsius for one hour.
k. No unauthorized person should be allowed access into the preparation room.
l. The lab technician should have a change of clothing (apron, cap and mask) and wear laboratory foot ware prior to entry into the dilutor preparation room.
m. The hands of the technician and the work table / laminar air flow unit (LAFU) should be sterilized by use of 70% alcohol.
n. Preferably surgical disposable gloves may be worn by the technician.
o. The buffer preparation should be done under a LAFU.
p. The composition of the buffer is as follows
    1. Tris - 24.20 grams
    2. Citric acid - 13.60 grams
    3. Fructose - 10.00 grams
q. Milli Q water / autoclaved distilled water – 736 ml is added and the same is mixed well.
r. The buffer is then autoclaved at 5 pounds pressure for a period of 10 minutes.

s. After cooling the PH of buffer is taken by use of a PH meter which has been standardized and has been put on at least 20 minutes prior to estimation of PH.
t. The Ph of buffer should be between 6.7 and 6.9.
u. When ever chemicals of a new batch / company are introduced care should be taken to test its quality prior to use.

Dilutor preparation

a. The autoclaved buffer is cooled. If not used immediately it should be stored in a refrigerator at 4 to 8 degree Celsius.
b. The dilutor can be prepared fresh on the day of collection early in the morning or on the previous day in the evening.
c. If prepared on the previous day it may be noted that antibiotics should be added only in the morning prior to use.
d. All hygienic measure as mentioned in the buffer preparation should also be employed during dilutor preparation by the lab technician and should be carried out under a LAFU.
e. The buffer prepared the previous day is taken in a one litre graduated measuring cylinder and to this 64 ml of Glycerol (AR grade from a reputed company) is added.
f. Glycerol can be warmed after measuring to increase its miscibility or added to the buffer itself prior to sterilization the previous day.
g. 200 ml of egg yolk is then added to prepare the extender / dilutor.
h. Eggs used for preparation of dilutor should be fresh and purchased from a known source to avoid mycoplasma / salmonella infections.
i. The eggs should be stored in a refrigerator after wiping with dry cotton.
j. Just prior to dilutor preparation eggs shall be wiped with 70% alcohol.
k. Egg yolk shall be separated from albumin using an egg separator onto autoclaved filter paper (Whatman filter paper. no 1) and rolled over to remove the entire albumin before addition to the buffer.
l. Finally antibiotics are added to the dilutor (benzyl penicillin 10 lakh IU per litre and dihydro streptomycin 1 gram per litre of diluent).
m. PH of the final diluent should be taken daily and recorded
n. The glass stopper is put on the measuring cylinder and diluent mixed gently and transferred to a conical flask.
o. A sterilized magnet is put into the conical flask, covered by aluminium foil and then placed on a magnetic stirrer for a period of 30 minutes.
p. After the preparation of dilutor all the materials should be sent for cleaning and sterilization.
q. The work area should again be sterilized by use of 70% alcohol.

Extension and Evaluation of semen

a. Is done in the processing room which has been well fumigated with formalin regularly and aerated prior to commencement of work.

b. Only authorized personnel shall be allowed access into the processing room.
c. The room temperature shall be maintained at 20° C by use of split air conditioners.
d. No staff from the semen collection group / AV preparation room and visitors shall be allowed in the processing room.
e. All staff working in the processing room should wear laboratory apron, mask, cap and disposable rubber gloves in the laboratory.
f. Reusable clothing materials should be washed, cleaned and sterilized on a daily basis.
g. The work tables / surfaces / LAFU and hands of operator should be sterilized by use of 70% alcohol prior to commencement of work.
h. The semen in a collection tube capped with aluminium foil, suitably identified by the collection team is placed in the water bath of 34° C in the pass box.
i. As soon as the semen is received it is observed for colour, and presence of visible contaminants.
j. The volume of semen along with the breed and bull number is recorded.
k. The sperm concentration is assessed in a bovine photometer which has been standardized and validated regularly at least once in six months by hemocytometer readings.
l. A fresh cuvette is used for each sample / ejaculate.
m. Sampling tube should also be changed in the dilutor unit for each ejaculate and never reused.
n. Initial dilution of semen with diluent maintained at 34° C in a thermo controlled water bath is done for all samples showing concentration of greater than 500 million / ml
o. Semen samples with concentration below 500 million per ml are discarded
p. First and second ejaculates of the same bull are processed separately and the processing should be done without delay at any stage.
q. In case of Buffalo bulls the mass activity of semen is also examined prior to dilution.
r. Mass activity is observed by placing a drop of neat semen on a warm slide without a coverslip and observed for the swirls / waves and graded on a scale of 0 to 4 depending on the vigorosity of the waves.
s. As static ejaculates that show no mass activity seldom freeze well they are discarded.
t. The initial motility of semen sample is done by taking a drop of diluted semen (by use of a micropipette and a tip) onto a warm slide (maintained on a slide warmer at 37° C) and a coverslip (18 mm square or round) is put on the drop.
u. Place the slide on the biotherm (warm stage at 37° C) of the phase contrast microscope and examine the motility (movement of individual sperms) at a magnification of 200 times (10X eyepiece and 20X objective).
v. Semen samples selected for freezing should have a minimum of 70% initial motility.

w. Once accepted for further processing the semen sample may be finally diluted by addition of calculated quantity of diluent maintained at 34° C into a sterilized beaker / conical flask / centrifuge bottles of suitable capacity so as to maintain a sperm concentration of 20 million spermatozoa per 0.25 cc straw.
x. All this work should be done under a LAFU.
y. All used glass wares are immersed in a tub containing a neutral reagent to prevent drying and ease in cleaning.

Printing, filling and sealing of straws

a. Sterilized French mini straws (0.25 cc) of reputed companies are used.
b. Printing of straws is done by use of an automatic straw printing machine / Jet printer and the straws are imprinted with the details of bull number / name / breed / name of the organization / batch number / year etc.
c. The clarity of printing on all straws should be checked.
d. Unused straws shall be repacked air tightly under the LAFU and sealed in a sealing unit for future use.
e. Using a fully automatic filling and sealing machine the diluted semen is filled in French mini straws under a LAFU using sterilized nozzles and fresh rubber tubing's.
f. The rubber tubing should not be reused
g. Sufficient number of sets (3 nozzles with rubber tubing and straws) are prepared in advance based on the number of bulls under collection.
h. The diluted semen is gently mixed before filling to get uniform number of sperms in all the straws.
i. Air space of 1 cm and the quality of sealing of straws at laboratory end should be checked at random
j. All processing work including filling is done at room temperature (20° C) and there should be no delay at any stage of processing of semen
k. After filling and sealing of straws the unit is cleaned with 70% alcohol
l. All glass wares are immersed in neutral reagent in a basin for washing and sterilization.

Racking of filled straws and equilibration of semen

a. Once filling and sealing of straws is done the straws are immediately racked on racks by use of the ramp and racks and then placed in a cold handling cabinet (CHC) placed in the freezing room.
b. After the racking of straws (each rack has 175 straws) the CHC is put on so as to attain a temperature of 4 ° C
c. The straws are maintained at this temperature for a period of 4 hours
d. This pre freeze storage period is termed as equilibration period. During this period the sperm cells become permeated with glycerol and an ionic and osmotic equilibrium is established with the media. This period also helps the spermatozoa to gain resistance prior to freezing stress.

Freezing of straws

a. The filled and sealed straws can be frozen by two methods, namely:
    1. Static vapour freezing
    2. Forced vapour freezing

Static vapour freezing

a. This is carried out in wide mouthed liquid nitrogen containers (LR 320 or LR 250)
b. The straws on racks are placed on the grill which is kept 32 cms away from the brim (mouth) of the container
c. Level of Liquid nitrogen (LN) is maintained just above the surface of the grill
d. A ply wood sheet is placed on the mouth of the container for settling of the LN vapours
e. Three racks of 175 straws each is placed on the grill after settling of vapours for a period of 10 minutes
f. The height of the freezing racks is 4 cm from the grill where the LN vapour has a temperature of minus 180 ^{o} C
g. After freezing the straws are collected and plunged into precooled goblets for storage.
h. Care needs to be taken during freeing since the nitrogen gas has a low heat capacity per unit volume (1 / 1500 of alcohol) whereby a small amount of heat will warm the gas a great deal and thus disturb the static vapour equilibrium drastically.

Forced vapour freezing

a. This is carried out using a programmable bio freezer
b. The rate of freezing is highly precise and the program is fed in the computerized freezer
c. The rate of fall of temperature is as follows:
    1. The machine is put on at 20^{o} C (room temperature) and brought down at @ 10 degree C per minute till 4 ^{o} C.
    2. Here it is paused automatically for transfer of straws from the CHC.
    3. Each batch can freeze 30 racks of 175 straws or 5250 straws.
    4. A straw is cut and placed on the thermocouple to record graphically the fall of temperature within the straw.
    5. The machine is then closed, once the temperature is stabilized at 4 ^{o} C it is started again.
    6. The rate of fall of temperature is @ 5^{o} C from 4 ^{o}C to minus 10^{o}C.
    7. From minus 10 degree C the rate of fall has been programmed at @ 40 degree C per minute up to minus 100 degree C.

8. Finally from minus 100 degree to minus 140 degree C at @ 20 degree C per minute.
9. After 140 degree the freezer is opened and the straws are removed from the racks and placed into pre cooled goblets and then plunged into LN at minus 196 degree C.

d. Samples as per requirement are drawn after freezing to assess post thaw motility and other quality control tests as per MSP.

Post thaw evaluation

a. After 24 hours period in LN the frozen semen straws are re evaluated for post thaw motility
b. One straw should be thawed at a time
c. Each straw is removed from the container by using a forceps
d. Jerk the straw once to remove any LN in the factory seal end and put into the thawing unit or water bath maintained at 37 degree C and thaw for a period of 30 seconds.
e. Evaluate the post thaw motility (PTM) by repeating the procedure for 2 to 3 straws per bull / batch
f. Forward motility under the phase contrast microscope (200X) with a warm stage / bio therm is observed
g. Only samples that show a PTM of 50% and above should be preserved
h. Those below 50% PTM should be discarded.

Quarantine of semen doses

a. The frozen semen accepted for long term storage should be held in the laboratory for a period of one month as a quarantine mechanism
b. During this period if there is no out break or incidence of disease the semen is passed on for dispatch / sale / bulk storage.

Laboratory hygiene

a. The lab is wet mopped with disinfectant solution daily after the completion of the processing work before closing for the day
b. The work tables , all surfaces of CHC and other horizontal surfaces are mopped clean with 70% alcohol
c. Formalin humidification is done twice a week on Wednesday and Saturday by use of 12 ml formaldehyde solution in 100 ml of water per cubic meter of lab area including the AV preparation room
d. The bacterial load may be monitored regularly to see the efficacy of sterilization by exposing agar plates in all rooms of the lab
e. All laboratory footwear and protective clothing should be cleaned and washed daily prior to use the next day.

Chapter - 4

Standard Operating Procedures for Post – Thaw Viability

Standard Operating Procedures for Post – Thaw Viability

The post-thaw Viability of spermatozoa is dependent primarily upon recovery from freezing without membrane damage. The following parameters are assessed.

1. Percent of Progressively motile cells.
2. The rate of progression.
3. Percent intact acrosome.

1. Motility Examination

Objective:

Examination of motility in post-thaw frozen semen sample.

Discussion:

Sperm motility must be considered one of the most important parameters in evaluating the fertility potential of semen specimen. The type of movement will also influence fertilizing capacity. Thus sperm swimming in tight circles cannot readily parts through the utero-tubal junction, and only straight swimmers succeed in fertilizing ova. Vigrous beating of the sperm tail is necessary for penetration of the sperm head through the corona radiate to fertilize the ovum.

Assessment of sperm movement

Quantitative assessment

For the purpose of semen analysis, sperm motility may be recorded as the "Percentage motility" i.e. the percentage of the total sperm that are showing movement. The estimation of percentage motility should be performed immediately after thawing. Each estimation of motility should be performed in duplicate and the mean result recorded.

Qualitative assessment

The quality of sperm movement is usually graded according to the type of movement made by the largest proportion of the total sperm. The grading used can be numerical or descriptive, if numerals are used, sperm movement is graded from 0-5. The scoring system recommended is as follows.

Definition	Description
0- None	No movement
1- Poor	Slight tail undulation or vibration without Progression
2- Fair	Slow progression, including stop and start motion.
3- Moderate	Steady progressive motion at moderate speed
4-Good	Rapid progressive motion
5-Excellent	Very Rapid progressive motion in which cells are difficult to follow visually.

Method:-

Slide Technique:-

After thawing the Frozen semen straws in 370C water for 30 see, the lab end is cut and straw is emptied in a 2ml test tube, keep the test tube in water bath for 5 minutes. A very small drop is should no bubble in the similar otherwise there will be transferred on clean warm slide cover with a cover slip[18mm] care should be taken on over estimatean of motility Examine the smear under the phase-contrast micros cope under 10xor 20x.motility based on progremive motile sperm is estimated on the nearest 5 percent In general 50% point thaw motility as a minimum acceptable motility for frozen semen. Incubation test for Frozen thawed.

The thawed semen is kept in the water bath and every half an hour the motility is estimated for minimum two hours. Good quality semen will sustain motility upto 25% to 30% even after 2 hours of incubation. Every time a thin smear is made on a clean grease free slide for the study of percent intact acrosome (PIA).

Computer Assisted semen analysis (CASA)

An objective method of evaluating the sperm movement with the help of this system a detailed study can be made on the number of motile and non-motile sperm and the percentage of motile sperm. It also calculates the mean path velocity and draws a distribution of his togram of the speed of individual spermat ozoa in microns per second. The mean straight line velocity is calculated and from these figures the mean linearity is calculated. The linearity is an empirical assessment of the “straightness of the progress of sperm and thus is a function of the commonly known as progressive activity. Lateral head displacement and beat/cross frequency is also examined.

Material required

1. Micro glass slide, round cover Slip (18 mm)
2. Water bath, test tube stand, Test tube 2 ml
3. Slide warmer, Forceps, Scissor
4. Tissue paper, glass marking pencil

2. Percent Intact Acrosome (PIA)

Objective

Determination of PIA in a Post-thaw sample.

Discussion

The acrosome covers about 60% of the anterior portion of the nucleus. Acrosome can be detached from the sperm under the influence of different physical and chemical factors. The enzyme localized in the acrosome determines the sperms penetrating and fertilizing Capacity. Optimum fertility depends on the acrosome being structurally and biochemical intact.

Determination of the PIA is a morphologic method of measuring post-thaw viability which is highly correlated to fertility.

Method

A. Staining Technique (Giemsa method)

A thin smear of semen is made on a clean grease free slide. After drying the slide immersed in 5% formaldehyde (37%) for 30 minutes at 37oc. Then the slide is washed in running water and dried. From stock Gremsa stain solution, 3ml is taken and mixed with 2ml of sorensons phosphate buffer (PH-7.0) and 45ml distilled water. The fixed slide is kept in stain for 12 hours at 37oc, washed in water and dried.

Fresh stain should be prepared every time for staining the smear. Examine the smear under the oil immersion objective of the microscope and count total of 100 sperms recording number of sperms with intact altered and completely lost acrosome.

Giemsa stock solution

Giemsa powder – 3.8 gm
Methanol (AR grade) – 370 ml
Glycerol (AR grade) – 125 ml
Mix Giemsa powder in glass pastel and mortar by adding methanol slowly. Leave the solution for maturation for seven days at 37oc. During the period the stock solution is mixed well for a few minutes everyday by shaking the bottle.

Sorensons 0.1M phosphate Buffer PH-7

Stock solution A:

Disso 1e 13.069 gm of potassium phosphate, monobasic anhydrous (KH2 Pou) in one litre dist water.

Stock solution B:

Dissolve 14.0198 gm of sodium phosphate, dibasic, anhydrous (Na2 Hpo4) in one litre dist water.
Mix 17ml of stock solution A with 33ml of stock solution B.

5% Formalhyde solution

Formaldehyde (37%) - 13.5 ml
Distilled water - 86.5 ml

B. Wet technique

From the thawed frozen semen 0.5ml of the semen is mixed with 1ml of 0.2% glutaraldehyde + PBS or 1ml of 0.2% glutaraldehyde in three parts PBS and 1 part 0.15m Sodium Cocodyl place a drop of the solution on glass slide and cover with cover slip A thin smears is important. Slide is examined under oil immersion with DIC micro scope. Or phase contrast microscope Acrosome which are intact will have a dark ridge at the apex of the sperm cell. Swollen Acrosome lose the dark ridge and are not counted as intact Aerosome with broken membranes will usually show as faint rued line just ahead of the apex of the sperm nucleus. Non-intact acrosome lack a ridge or show a ruffled membrane separated from the apex of the nucleus.

Two hundred cells should be examined and the first and second counts of 100 should be compared, of these count are within 5% of each other no further count are necessary.

The Minimum values for satisfactory classification:

0 hr – 50% intact acrosomes Barath. A-D
2 hr – 35% intact acrosomes
4 hr – 50% intact acrosomes (select sire, U.S.A)

0.2% Glutaraldehyde in PBS

Add glutaraldehyde solution and mix in phosphate buffered saline (PBS, PH-7.2) to make the final volume 100 ml.

Phosphate buffered saline (PBS) PH 7.2

Dissolve 0.8gm sodium phosphate monobasic an hydrous (NaH2 P04) in 100 ml distilled water
Dissolve 0.947gm sodium phosphate dibasic anhydrous (Na2 Hp04) in 100 ml distilled water
Mix 30 ml NaH2P04 solution with 70ml Na2Hp04 solution. Dissolve 0.45g Nacl.

Material required

1. Giemsa powder (E.Merk), Glycerol AR, Methan or AR
2. Formaldehyde (37%), phosphate buffer,
3. Glutaraldehyde phosphate buffer saline.
4. Staining Jar, Glass slide, Coverslide, Text tube
5. Cede wood oil

3. An Assay to assesp the functional integrity of the sperm membrane (HOST)

Discussion

Two tests are available to evaluate the membrane integrity of the spermatozoa \, the supravital (viability, live-dead stain) and the Try po-osmatic swelling (HOS) test. However different aspects of spem membrance behavior are measured by each test. Vital stain meastires whether the membrance is physically broken, a sign of cell death. The HOS test evaluate whether an intact membrane is biochemically active. During the HOS test active spermatozoa when exposed to hypo-osmatic stress due to the influx of water, will undergo swelling and subsequently increase in volume to establish an equilibrium between the fluid compartment within the spermatozoon and the extra cellular environment. The plasma membrane surrounding the tail fibrers appear to be more loosely attached than the membrane surrounding the head,so that the tail region shows the swelling more clearly.

Method

a. Preparation of semen specimen

The two number frozen semen straw sample is thawed (37oc for 30 sec) pooled and slowly diluted 1:2 V/V in a 1.5 ml test tube using modified sodium citrate medium with 2% (V/V) fetal bovine serum (FBS) albumn. The specimen is centrifuged (37oc) for 8 min at 400 xg. The supernatant is discarded and the sperm pellet is slowly resuspended to 2ml modified sodium citrate medium with 2% FBS. The specimen is maintained in a water bath at 37oc and allowed to stabilize for 3 to 5 min. The percentage of motile cells is estimated microscopically at 37oc.

b. Preparation of Media

Hypo – osmatic solution is prepared by weighing the Fructose and sodium utrate as per the formula.

Ali 90 to of 1 ml can be prepared in the test tubes and stored at – 20oc for later use.

c. Procedure for HOS test

Add 0.1 ml of the specimen containing the spermatozoa with 1 ml of hypo osmatic solution. The solution is then incubated at 37oc for one hour.

d. Counting of spermatozoa

A drop is placed on the clean glass slide and covered with a cover glass and observed under phase-contrast microscope at x 400 magnification. A total of 200 sperm are counted in at least 5 different fields.

The percentage of swollen spermatozoa is calculated by the following formula.

$$= \frac{\text{number of sperm swollen} \times 100}{\text{Total number of sperm counted}}$$

The initial response of the sperm to the HO solution is a small enlargement of the tip of the tail or at the junction of the mid piece and tailpiece. Later the alteration are characterized by the presence of a swollen area at the tip of the tail (Fig b-d), a hairpin curvature of the tail (Fig c-e), a shortened and thickened tail (fig-f), or a swollen area that partly or completely enveloped the curvature of the sperm (fig de&g). There is good correlation between the percentage of motile spermatozoid and spermatozoa that reacted to the HOS test and between the percentages of sperm. With intact membranes and HOS relative sperm.

The sample to be treated well should show swelling of the entire tail region and should be accounted for more than 60%of the total swelling.

Preparation the Media

Sodium citrate 2 H20 - 7.35gm
Osmolality -150 mosmol
Fructose - 13.51 gm
Dist water - 100 ml
Ionic strength - 0.15

Material required

1. Centrifuge machine, water bath, phase contrast microscope.
2. Sodium atrate, Fructose, Fetal BSA,
3. Test tube, pipette, glass slide, cover slip

4. Study of Sperm Morphology

Objective

Examination of morphology in post-thaw frozen semen sample.

Discussion

The identification of different types of abnormal spermatozoa in semen is very important part of semen analysis. The many structural abnormalities that may occur in sperm are known to be associated with infertility.

I. Classification according to causative reason

i) Primary abnormalities: Macro head, Micro head, dwarf head, pyriform head, double head, double mid piece, knobbed head, abial head.

ii) Secondary abnormalities: Detached head, free head, coiled mid piece, proximal droplet, distal droplet.

iii) Tertiary abnormalities: Coiled tail, bent tail, acrosome malab formalities.

II. Classification according to affected portion of spermatozoa

1. Spermatozoal head abnormalities.
2. Spermatozoal mid piece abnormalities.
3. Spermatozoal Tailabrormatities.
4. Acrosome abnormalities.

a. Wet Preparation (Formal saline method)

1. Count 200 sperms in phase contrast microscope (10x1000 magnifications)
2. Take a sterilized 5ml Borosil glass tube with screw cap.
3. Add 2-3 drops of semen to about 5 ml of buffered formal saline, (warm to body temperature).
4. Gently shake and incubate for 10 to 20 minutes at 37oc
5. Put a drop of the sample on clean glass slide cover with cover slip and examined under the microscope in zig-zag manner.

b. Stained preparation (Eosin – Nigrosin / Aniline stain)

1. Count 100 sperm in phase contrast microscope [10x1000 magnifications].
2. Place 1-2 drops of the nitrosin solution (warmed to 37oc) and a small drop of semen at one end of a glass slide and mix with another slide Spread the smear on slide.
3. Counter stain with eosin solution.
4. The sperm will be seen against the dark background of nigrosin.
   (Note in place of Nigrasin, Aniline, can also be used)

Permissible value for semen assessment

(American society of Theriogenology)

Classification	**Percent Abnormal spermatozoa**		
	Primary(%)	**Total(%)**	**Score**
Very good	10	25	40
Good	10-19	26-39	25
Fair	20-29	40-59	10
Poor	29	59	3

Permisiable value for semen arsessment (As per lagerlof)

Head abnormalities –

Young bulls –10%
Old bulls – 20%
Proximal droplet-5%
Mid piece abnormalities- 5%
Tail Abnormalities- 5%

Formal saline

Stock solution A:

1. Na2 HPO42H2O –21.682gms
   Add Dist water –500 ml

2. KH2PO4 –22.75gms
   Add Dist water –500ml
   Take solution A-200ml stock buffer A
   Take solution B-80ml

Stock solution B

1. Nacl –9.01 gm
   Add Dist water –500ml
   Stock buffer solution A - 100ml
   Stock solution B -150ml
   Formaline (37%) -62.5ml
   Dist water -500ml

Or

2. NA2HPO42H2O - 6.19g
   KH2PO4 - 2.54g
   Formalin (37%) -125.0ml
   NaCl - 5.41g
   Add distilled water upto1000ml

Eosin Nigrosin Stain

Sodium citrate dihydrate	-3gms
Dist water	-100ml
Eosin B	-1gm
Nitrogen	-5gm

Eosin –Aniline Blue stain

Eosin B-1gm
Aniline Blue –4gm.
M/8 phosphate buffer –100ml
Stains are dissolved in the buffer heating it at 850c Do not boil.

M/8 Phosphate buffer

Dissolve 1.720gm potassium phosphate monobasic anhydrous(KH2PO4) in 100ml glass distilled water, Dissolve 1.776gm Sodium phosphate, dibasic anhydrous (NA2HPO4) in 100ml glass distilled water.

Mix 28.5ml KH2PO4 Solution with 71.5 ml NA2HPO4 Solution Examine ph which should be about 7.2.

Material required

1. Eosin blue (water soluble) Nitrogin, Anilineblue (Gurr company marked by E.mark).
2. KH2PO4, Na2PO4, NaCl,
3. Test tube 5ml glass slide, cover slip with screw cap, coupler Jar.

5. Study of Micro – Organism in frozen semen straws.

Objective

To study the method for enumeration of living Aerobic Micro – Organism in frozen semen straws.

Discussion

Extended semen may contain organism from the bull or can be added by contamination during processing. Many organisms can survive in frozen semen. Bacilli, diptheroids, micro cocci, collform organisms, strptococi, staphylococci, Pseudomonas, molds, yeast, mycoplasm, and viral agents have been identical field in bull semen. Many of the organism present are pathogenic.

Other organism which are contagious and can infeet courst through contaminated semen include Brucella abortus, camplyobactor, trichomonas, foctus, and so on.

There is considerable variation in seminal processing procedures among A.I Organisation, Consequently, O.I.E. have recommended that processed semen should not contain more than 5000 CFU per sample including common bacteria, fungi, Moulds, yeasts per units of semen.

a. Media Preparation

1. Preparation of plate count agar

a. Use conical flask 2-3 times larger than the volume of the media to be prepared.
b. After dissolving the weighed medium in the required quantity of distilled water boiled the medium till it completely dissolved. Care should be taken not to heat the medium excessively. The water should be double glass distilled and deionized.

2. Adjustment of pH

a. The pH value of reconstituted dehydrated culture media prepared with distilled water shall produce equivalent value with the prescribed on the label at a temperature of 250c measurements of pH shall be made using a pH meter.

b. pH measurement and correction of the pH should be carried out at 250c with solid as well as liquid culture media. The pH should be adjusted to the value specified and it is corrected by adding 1N or 1/10 N Hydrochloric acid or sodium hydroxide solution to a sample of known volume taken from the reconstituted culture (Say 50 to 100ml). Finally, after calculation the required acid or alkali is added in the remaining prepared culture medium. The pH of plate count agar should be 7.4 + 0.1

3. Sterilization of Medium

a. Media should be sterilized as disected on the label.
b. Transfer the Agar into conical flask (500ml). The volume of media should be half the capacity of the conical flask.
c. Sterilize by autoclave at 121$\pm$10c temperature, 15 lbs pressure for 20 minutes
d. If the medium is to be used immediately, coot it to 45 $\pm$ 0.50c in the water bath Otherwise, before beginning the microbiological examination, completely melt the medium in a boiling water bath, cool to 45$\pm$0.5^{0}c in an other water bath (Add 10% (By Volume) inactivated and sterilized (by ultra filtration (0.2mm filter) bovine serum. – optional)

b. Dilution Media

Peptone salt solution Composition

Enzymatic digest of casein - 1.0 gm
Sodium Chloride (Nacl) - 8.5 gm
Water - 1000ml
Tryptone bacteriological – lobe chemic, Art No – 6405

Preparation

Dissolve the components in water, by heating if necessary.

If necessary adjust the pH so after sterilization it is 7.0+0.2 250c.

This solution is placed in 10ml screw capped tubes each containing 3.6 or 9 ml of solution, then it is sterilized in the autoclave for 20 minutes at 1210cs

c. Method of preparing dilutions of semen

1. Thawing

Prepare one tube containing 3.6 ml dilution media and four tubes containing 9ml.
The samples of semen are kept in liquid nitrogen (30 days) until required. They are then thawed in a water bath at 370c for 2 minutes before being transferred to cultures. Two semen samples from the same ejaculate are required for each analysis.

2. Dilution

After thawing, rapidly dry the semen container then disinfect it with 70% ethyl or 98-99% isopropyl alcohol.

After opening, transfer the two semen samples into a sterile tube. Measure exactly 0.4 ml of semen and place this in the tube containing 3.6 ml of dilution media (1:10 dilution). Mix the contents by stirring (Vortex apparatus), then prepare dilutions of 10-2 to 10-5 in four tubes each containing 9ml of dilution medium / 1ml + 9ml).

d. Inoculation and incubation

Take 0.5 ml of each dilution and introduce under sterile conditions (Laminar flow table with Bunsen burner) into each of four petri dishes, 9-10cm diameter. Add to each petri dish about 15 ml of counting agar cooled to 450c Mix by circular agitation. Allow to solidify on a level surface.

The time elapsing between the end of the preparation of the initial suspension and the moment when the medium is poured into dishes shall not exceed 15 minutes. Invert the prepared plates and place them in the incubator maintained at 370c + 10c for 72 h

Control plates

Inoculate and incubate two control plates in parallel with the operations by using 0.5 ml of diluent.

e. Interpretation of results

1. Examination of Control plates

In all cases, carryout an initial examination of the control plates to determine whether colonies are present within the medium. If colonies are present, discard plates and the plates containing the test sample and recommence the procedure.

If colonies are not present proceed to an examination of the plates containing the test sample.

2. Counting

After 72 hours of incubation at 370c the colonies are counted by means of the colony counting equipment or using the naked eye.

Count only well distinguishable colonies, which have grown within the medium and on the surface of the medium. Reject any plate in which more than half of the surface is overgrown. Select for the purpose of counting the dilution which contains between 30 and 300 colony forming. Units (CFU) perdish.

f. Expression of the results

Record the number of CFU counted. Multiply by the dilution factor. Express the results as the number of micro organism or CFU per sample or per ml of semen.

g. Method of calculation

N = C X D
Where,
N = Number of Colony forming Unit per ml or per straw
C = Number of Colony Counted
D = Dilution factor

If the colony forming Unit has to be expressed per straw we have to divide by 2
If the Colony forming Unit has to be expressed per ml we have to multiple by 2.

Media

Plate count Agar (Himedia Lab Cat.No.M091)

Casein Enzymatic Hydrolysate	gms/litre

Material Required

1. Hot air oven, Autoclave, Pressure cooker (10ltrs), Incubator up to 70o C.
2. Water bath capable of being maintained at 37+ 0.5oC and another at 45 + 0.5oC.
3. pH meter, electric, accurate to + or – 0.1 pH unit at 25oC.
4. Laminar airflow table, Bun son burner.
5. Colony counter of an illuminated base with a dark background filled with a magnifying lens to be used at a magnification of 1.5 diameters & a mechanical or electronic digital counter.
6. Automatic dispenser pipette with disposable Tip.
7. Vortex mixture.
8. Test tube screw cap 10ml capacity.
9. pipettes (not blow out pipettes) having capacity of 25 ml,1ml,graduated in division of 0.1,bulb rubber.
10. Conical flask (Borosil) 1000ml, 500ml, 250ml.
11. Glass (Borosil) petridish of diameter 90 to 100 mm.

6. Validation of photometer

Objectives:

Validation of photometer by haemocytometer.
(Concentration measurements)

Discussion

Sperm concentration describes the number of sperm in millions that are present per unit volume of seminal fluid.

Time to time the concentration of sperm is measured from the cuvette to asses the accuracy of the photometer.

Diluents

Either of diluents is used for diluting the sample.

1. Distilled water or
   Distilled water-50ml
   2% Eosin –1ml (to provide a background).

2. Buffered 3 % Formal Saline
   NaHCO3-5.0gm
   Formalin (35%) – 1ml
   Distilled water – To Produce final volume of 100 ml

To produce a stain 5 ml of saturated aqueous gentian violet may be included in the final volume.

Sample preparation

The cuvette is homogenizes 3 times and diluted with diluent at the rate of one volume of cuvette sample for 1 to 4 volumes.

Preparation of the counting chamber

Production of Newton ring on cover slip is important to achieve depth.
After applying the saliva on the lateral edges of the coverslip the coverslip is slided on the neubaer counting chamber with thumb pressure with a back and forth movement until the slide sticks.

Charging of chamber

20 ml drops are drawn with the micropipette of the sample & both the chamber are charged.
Allow the sperm cell to settle for 10 to 20 minutes but the chamber should not be allowed to dry (can be kept in the refrigerator)

Counting

Count under a X 400 magnification preferably with phase contrast. The stage of the microscope should be cold because if the drop retracts, the sperm will concentrate and the result will no longer be correct. Count every sperm (head is the marker) in five large squares containing 16 small squares each.

Count the spermatozoa whose head is inside the square, where the flagellum is, plus the ones whose head is situated on the northern and western limits.

Do not count the spermatozoa whose head is outside the square, wherever the flagellum is and the ones whose head is located on the southern or eastern limits.
Both the chamber is counted and then average is taken.
The concentration per ml of neat semen is then calculated.

Characteristics of the neubuaer chamber

(Depth) volume above the grid	1/10 mm3
Quantity of large squares	25 (big) x 16 (small) chambers
Quantity of small squares	400 (1 sqmm)

Characteristics of the sperm dilution

1. Dilution of neat semen for reading with the photometer.
   20 ul semen + 3980 ul Nacl
   20/ 20+3980 = 20/4000=1:200

2. Dilution for counting
   1 ml cuvette solution + 1 ml diluent 1: 2

Characteristic for Counting

Quantity of large squares counted per chamber = 5 (Four corner and one middle)
Total number of spermatozoa counted on 2 number chambers=300
=150(average)

Calculation

Area of 80 small squares = 1/400X80=1/5 sqmm
Volume in80 small squars=1/5X1/10=1/50Cmm
No of sperms in 1/50 cu mm area=N
No of sperms in 1 cu mm area = Nx50=50N
No of sperms in 1 Ml of semen=50Nx1000
Dilution factor=50Nx1000x400
Total no of sperms/ml =Nx20xmillion
=150x20x106
=3000X106

Material required

1. Improved Neubourer chamber
2. Pipette 1 ml , Micropipette
3. Test tube

7. Study of Concentration in Frozen Semen

Objective

Estimation of sperm concentration in frozen semen.

Sample preparation

Thaw about 4-5 frozen semen straws in water bath at 37 ^{o}C for 30 seconds. Cut the straws from laboratory seal end. Empty the straws with a plunger slowly into the small test tube.

Preparation of counting chamber

As explained above.

Dilution of semen sample

Dilute 0.1ml of semen (after homogenisation) in 9.9 ml of diluent (Any diluent)

Dilution = 0.1/0.1+9.9 = 1:100

Charging of Counting Chamber

As explained above.

Counting

Count the number of spermatozoa present in four corner chambers and one middle chamber (16x5=80 small chambers)

Calculation

N = X x50x100 (dilutor) x 1000 (Calculation factor)
= X x106 ml

To calculate exact number in usable volume.

N = X X 106 x 0.48 (French medium)
= X X 106 x 0.23 (French Mini)